DNAJ proteins: more than just “co-chaperones”

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Chapter 4

DNAJB6 fails to prevent the nucleation of intracellular polyQ proteins mediated by extracellular purified polyQ-peptide fragments

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ABSTRACT

Huntington’s disease (HD) is associated with protein aggregates that also hallmark almost all the major neurodegenerative diseases known. The aggregate formation is thought to be initiated by small polyglutamine (polyQ) peptides, which act as “seeds” for aggregation of polyQ stretch containing proteins within the cell. It has also been suggested that polyQ peptides or aggregates can be released from cells and that this extracellular polyQ material next can enter the neighbouring cells. The infiltration of these extracellular peptides further acts as seeds for aggregation and hence a prion-like propagation of the disease follows. Here, we show that intracellular overexpression of DNAJB6 and various members of the HSPB family, which are established to be suppressor of polyQ aggregation initiated by intracellular expressed polyQ fragments, fail to prevent this “induced” intracellular aggregation by extracellular polyQ seeds. Our data not only confirm previous in vitro findings that DNAJB6 inhibits polyQ aggregation at a very early step, but also suggest that aggregate initiated or triggered by extracellular polyQ material requires handling different than that from aggregates initiated by intracellular polyQ fragments.
INTRODUCTION

Polyglutamine diseases (polyQ) are a set of neurodegenerative diseases (Huntington’s disease; HD, several spinocerebellar ataxias; SCA, and spinal bulbar muscular atrophy; SBMA) that are characterized by expanded glutamine (CAG) repeats beyond a certain threshold in different genes (1,2). The expanded polyQ stretch in the corresponding disease-related proteins has a propensity to aggregate through forming highly ordered β-sheet rich structures commonly referred to as “amyloids” (3,4). Although not yet precisely clear how, but aggregate formation is suggested to be the basis of disease initiation. How aggregate formation is initiated (and hence what determines the onset of neurodegeneration) is still not fully understood. However, several lines of evidence indicate that activation of proteases that subsequently cleave full length polyQ proteins, that themselves are not very aggregation-prone, into smaller fragments is required (5–7). These small fragments containing the CAG expansion next can act as aggregation seeds and nucleate the aggregation of other polyQ containing full length proteins (8–10).

Heat shock proteins (HSPs) by virtue of their function as molecular chaperones act as the first line of defence in against protein aggregation. By their binding to un- or misfolded proteins, they not only can facilitate proper protein folding but also assist in protein clearance (11–13). In dedicated screens for members of the HSP families that might be good suppressors of polyQ aggregation, a number of very effective HSPs were identified, especially within the DNAJ and HSPB family, with DNAJB6 and DNAJB8 to be the most potent (14,15). In particular, we found that DNAJB6 not only prevents polyQ mediated aggregation in cells (14), simple organisms (16) but also but also delays aggregation and disease initiation in mice (Kakkar et al., submitted). The remarkable potency of DNAJB6 was suggested to be related to its ability to bind to small polyQ peptides (“the seeds”), hereby prevent the initiation of the aggregation reaction (17). Indeed, DNAJB6 also directly suppressed aggregation of polyQ peptides generated internally in living cells (18).

Recently, it has been suggested that for polyQ diseases, like for many other neurodegenerative diseases, aggregates may be transmitted from cell-to-cell and spread throughout the brains in a prion like mechanism (19). Indeed, synthetic polyQ peptides form filamentous aggregates and can be internalized by mammalian cells in culture, and gain access to the cytoplasmic compartments where they can act as potential “aggregate seeds” (20,21).

In this study, we wondered whether the members of various HSP families that can prevent polyQ aggregation initiated by intracellularly expressed polyQ peptides also would be able to prevent aggregation induced by these extracellular-polyQ peptides. Unexpectedly, all the combinations of chaperones tried in this study failed to prevent the aggregation initiated by extracellular polyQ.
RESULTS

Extracellular Q-peptides can seed the aggregation of cytoplasmic Htt-polyQ proteins
As previously suggested, extracellular Q-peptide can be internalized in cells and trigger the aggregation of even non-pathogenic polyQ repeat proteins (21). To be able to test effects on chaperones on such a seeding effects, we first had to establish a reliable (semi) quantitative assay using our HEK293 FRTTO cells in which we can precisely regulate the induction of these chaperones (14). Here, the HEK293 cells were transfected with plasmids encoding the exon1 fragment of the Huntingtin protein (Htt) with 23, 53 or 74 Q-repeats fused to GFP (HttQ23-eGFP, HttQ53-eGFP and HttQ74-eGFP respectively). 48 hours after expression, HttQ23-eGFP and HttQ53-eGFP did not form any detectable aggregates whilst expression of HttQ74-eGFP had resulted in formation of protein aggregates that could be trapped as SDS insoluble material on cellulose acetate filters (Fig. 1A) or could be detected in the stacking of SDS-PAGE gels (Fig. 1B). The addition of extracellular Q-peptides (Q30 or Q45) not only worsened the aggregation of HttQ74-eGFP but also lead to the formation of intracellular aggregates of HttQ53-eGFP and of the non-pathogenic HttQ23-eGFP (Fig. 1A, B). Also, immunochemically the aggregates were visible for all the three constructs used with the addition of Q45 peptide (Fig. 1C). Aggregates in Fig. 1 were detected using a GFP antibody. As a control, we also used an anti-polyQ antibody 1C2, which confirmed the data with the GFP antibody (Fig. S1) and which also showed that the extracellular Q-peptide did not seed the aggregation of eGFP. This confirms that the Q-peptides act by nucleating the aggregation of (other) polyQ proteins, and not by aspecific overload of the cellular chaperone capacity of the cell (Fig. 1A & 1B).

Extracellular Q-peptides co-localize with aggregated cytoplasmic Htt-polyQ protein
To further ascertain whether extracellularly applied Q-peptides indeed are in contact with and hereby seed the internal polyQ repeat constructs, we used Alexa-550 tagged extracellular Q-peptides. Whilst inclusion of extracellular Q45 peptides without intracellular HttQ23-eGFP or HttQ74-eGFP could be detected (red signal), most HttQ74-eGFP and all HttQ23-eGFP aggregates were positive for the Q-peptides (Fig. 2), further suggesting that Q45 peptides can enter the cells and get access to the cytosolic compartment to act in a prion-like manner conferring a heritable phenotype on cells expressing the non-pathogenic homologous protein.

DNAJB6 fails to prevent extracellular polyQ-peptide mediated “induced” aggregation of Htt-polyQ constructs
Using the current setup, we next asked whether DNAJB6, as one of the most potent HSP family member to protect aggregation of intracellular polyQ fragments (14,17), also would be
DNAJB6 can’t prevent aggregation propagation

Fig. 1: Extracellular Q-peptide “seeds” aggregation of cytoplasmic Htt-polyQ proteins

(A) Filter trap assay of HEK 293 cell extracts overexpressing different Htt-exon1-polyQ lengths. Q-peptides were added to the culture medium during 48 hours. Serial five-fold dilutions were loaded on cellulose-acetate membranes and probed with anti-GFP antibody. (B) SDS-PAGE with stacking gel (showing aggregates) was performed to check for the expression of Htt-exon-1 polyQ constructs. (C) Representative confocal pictures of cells transfected with HttQ23-eGFP or HttQ74-eGFP (green) incubated without and with Q-peptides. Q45 was added to the medium during 24 hours. DAPI staining is shown in blue. Bar represents 20μm.
able to prevent the polyQ aggregation induced by these extracellular-polyQ peptides. 1μM of extracellular polyQ-peptide was added to cells one day after transfection with the HttQ23-GFP or HttQ74-GFP and the molecular chaperones and the amount of aggregates were followed in time in which DNAJB6 was either turned on or not (Fig. 3). No aggregates were detected for cells expressing HttQ23-GFP alone. However, the addition of Q-peptides already leads to the co-aggregation with HttQ23-GFP within a few hours. To our surprise, DNAJB6 completely failed to prevent extracellular polyQ peptide mediated induced aggregation of HttQ23-eGFP even at the early time points although it completely suppressed formation by intracellular HttQ74-eGFP aggregates without addition of extracellular polyQ peptide (Fig. 3).

Most of the members of different chaperone families fail to prevent the polyQ-peptide mediated “induced” aggregation of Htt-polyQ constructs

Since we did not find any effect of DNAJB6 on seeding effect of extracellular polyQ peptides, we wondered if other chaperones might work in this setup. As DNAJB6 is thought to act at an early stage of aggregation initiation by peptides, we argued that its failure to act on seeding via extracellular peptides might be due to the fact that these enter the cells after released into

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Fig. 2: Co-localization of extracellular polyQ peptides with cytosolic Htt-polyQ protein

Representative confocal pictures of HEK293 cells transfected with HttQ23-eGFP or HttQ74-eGFP (green) incubated with Alexa550 (red) labelled extracellular Q45 polyQ-peptides. Overlay shows that most of the extracellular polyQ peptide co-localize with HttQ23/Q74-eGFP. DAPI staining is shown in blue. Bar represents 20μm.
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Fig. 3: DNAJB6 is unable to prevent the “induced” aggregation of HttQ23/Q74-eGFP via extracellular polyQ peptides.

(A) Filter trap assay of cell extracts at indicated time points. Serial five-fold dilutions were loaded on cellulose-acetate membranes and probed with anti-GFP antibody. For control condition, co-transfection of HttQ74-GFP +/− DNAJB6 is shown without the addition of extracellular peptide. (B) Western Blot assay of cell extracts at indicated time points. Nitro cellulose membranes were probed with the indicated antibodies. (C) Representative confocal pictures of cells co-transfected with DNAJB6 and HttQ23-eGFP or HttQ74-eGFP (green). One day after transfection the cells were incubated without and with Q45 added to the medium during 1 day. DAPI staining is shown in blue. Bar represents 20μm.
the cytosol in a fibrillar state that is beyond control of DNAJB6 (17). Based on our previous work (14,15,22), we selected a number of chaperones and tested their effect on intracellular aggregate nucleation by extracellular polyQ peptides. Strikingly, none of them showed even the slightest reduction in polyQ aggregation in the current setup (Fig. 4A, left column).

Since most of the selected chaperones are thought to work within the context of the central Hsp70 machinery, we also combined their overexpression by co-expressing with HSPA1A, the main stress inducible Hsp70 family member. Yet, neither HSPA1A overexpression alone nor in combination with any of the selected chaperone members protected against induced aggregation of the HttQ23-GFP by the extracellular Q45 peptide (Fig. 4A, right side panel).

**DISCUSSION**

Although more and more evidence is indicating that in some neurodegenerative diseases like Alzheimer’s disease and Parkinson’s disease aggregates may transfer from neuron to neuron and show have a prion-like infectivity (23,24) this is still less clear for polyQ diseases like Huntington’s disease. Yet, the fact that synthetic polyQ peptides form filamentous aggregates and are internalized by mammalian cells in culture, and gain access to the cytoplasmic compartments where they can act as potential “aggregate seeds” at least suggests that also polyQ disease may spread in a prion-like manner (20,25).

In the current study, we confirmed that extracellular polyQ peptides indeed can infiltrate cells and initiate the aggregation of the intracellular polyQ proteins, even those that normally would not aggregate supporting that a prion like-propagating may also play a role in diseases like HD.

Strikingly, however, the seeding of intracellular aggregation by these added extracellular Q-peptides is beyond the control of chaperones that previously were shown to have potency to prevent intracellular polyQ aggregates. This includes DNAJB6 that was shown to be most potent suppressor of polyQ mediated aggregation (14). The failure of DNAJB6 in preventing this nucleation effect might be attributed to the fact that DNAJB6 was mainly found to prevent the initiation of “seeding effect” mediated by polyQ peptides and not on aggregate elongation by preformed aggregates (14,17). The external polyQ peptide probably already enters in a fibrillated state (so preformed intermediate aggregate moiety), which renders DNAJB6 incapable to handle it and hence the aggregation cascade takes place. Our findings that the aggregation process indeed is so fast (see Fig. 3) indeed suggest they must have entered the cells as pre-seeds since aggregate initiation by Q-peptides are usually characterized by long lag-phases (17). Inversely, these results thus indirectly also validate the point that DNAJB6 is indeed an “early stage” polyQ aggregate mediator.
DNAJB6 can't prevent aggregation propagation

Fig. 4: Members of different chaperone families fail to prevent the polyQ-peptide mediated “induced” aggregation of Htt-polyQ constructs

(A) Filter trap assay of HEK 293 cell extracts co-expressing HttQ23-eGFP and different chaperones with (right side) or without (left side) HSPA1A with 1uM of Q45 peptide added. As a control HttQ23-eGFP was co-expressed with HSPA1A without any peptide (upper most row). Serial five-fold dilutions were loaded on cellulose-acetate membranes and probed with anti-GFP antibody. Post-lysis control (PLC) was also used (preparation described in the method section).

(B) SDS-PAGE with stacking gel (showing aggregates) and soluble fractions was performed to check for the expression of Httexon-1 polyQ constructs. Corresponding antibodies were used to check for the expression of various chaperones.

The seeds also seem to be beyond control of HSPB1 and HSPB5 of which it had been suggested that they may delay fibril elongation in vitro (26), although it must be stated that we never found these HSPB members to be very effective in delaying aggregation initiated intracellular polyQ fragment (15). The failure of DNAJB2 to act on aggregate seeding
DNAJB6 can't prevent aggregation propagation by extracellular peptides or fibrils suggest that also chaperoning the pre-seeds to the proteasome, in which this chaperone is thought to be involved (27,28), may not be possible. Maybe most surprisingly, chaperones like HSPB7 or the HSPB8/BAG3 complex that reduced intracellular polyQ aggregation by stimulating aggregate clearance through the autophagic machinery (15,29) were also ineffective and even overexpression of the FOXO1 that induces an entire repertoire of chaperones, proteases (Ying et al., manuscript in preparation) and that enhances autophagy (30,31), could not prevent aggregate formation initiated by extracellular peptides (Fig. 4A & 4B).

Together, this implies that if cell-to-cell transmission indeed plays a role in the progression of CAG repeat disease, upregulation of endogenous protein quality control systems may no longer suffice. Interestingly, we recently found in a cohort of SCA3 patients that whereas age at onset was clearly associated with CAG repeat length (and thus intracellular aggregation initiation), the rate of progression of the disease was not (Verbeek et al., manuscript in preparation). Together with our demonstration that DNAJB6 can actually delay the onset of disease in a Huntington mouse model (Kakkar et al., submitted), this would imply that initiation of disease (CAG dependent, chaperone dependent) and progression of disease (cell-to-cell transmission, immune response) may be partially independent, albeit that these are still interrelated processes.

MATERIAL & METHODS

**Cell line, culture and transient transfections**

Hek293 stably expressing the flip-in expression vector and a tetracycline (tet) repressor (Flp-In T-REx HEK-293, Invitrogen) are grown in DMEM (Invitrogen/Gibco, cat no 41966-052) supplemented with 10% foetal bovine serum (Greiner bio-one, cat no 758093) and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, cat no 15140-163). Furthermore they are kept under selective pressure to retain the tetracycline repressor and the Flp-in recombination site by adding 5 µg/ml Blasticidine (Invitrogen, cat no A11139-03) and 100 µg/ml of Zeocin (Invitrogen, cat no R250-01) to the culture medium. Cultures were maintained at 37°C and 5% CO2 in a humidified incubator.

For transient transfections, cells were grown to 50-60% confluence in 35 mm-diameter dishes coated with 0.001% of poly-L-lysine (Sigma, P8920) and/or on coated coverslips for (confocal) microscopy analysis. Cells were transfected with a total of 1µg DNA using Lipofectamine (Gibco) according to the manufacture instructions. To induce expression of the pcDNA5/FRT/TO vectors tetracycline was added to the medium in a final concentration of 1 µg/ml (Invitrogen cat no 550205). Controls are carried out without the addition of tetracycline.
Gene cloning

For the experiments plasmids were used driving the expression of a fragment of exon1 of Huntingtin with different sizes of polyQ fused to eGFP. peGFP-HttQ23 and peGFP-HttQ74 were kindly provided by David Rubinsztein, Cambridge, UK. Tetracycline inducible V5-tagged pcDNA5/FRT/TO plasmids encoding for the expression of several heat shock proteins were constructed as described in Hageman et al., 2010 (14).

Addition of Q-peptides

PolyQ-peptides (K2Q30K2, K2Q45K2) were obtained from R. Melki, Gif-sur-Yvette, France and were generated as described in (21). Stock solutions of Q30 and Q45 peptides with a concentration of 100 µM in Hepes buffer (Hepes 20 mM/NaCl 150 mM/pH=7.5) or tris buffer (tris-HCl 20 mM/NaCl 150 mM/pH=7.4) were kept at 4°C. Prior to addition to the cell culture the stock solution was mixed thoroughly by vortexing and pipetting repeatedly. Sonication appeared to reduce the co-aggregation. Addition to the culture medium to a final concentration of 1µM was done one day after transfection with the HttQ23-GFP and the molecular chaperones. For microscopy Q45 peptides tagged with Alexa 550, dissolved in tris buffer were added to the culture medium on the coverslip to a final concentration of 1µM. For most experiments the incubation time with the Q-peptides was 24 hours in the incubator at 37°C and 5% CO2.

Post-lysis control

Since the Q-peptides tended to co-aggregate very fast with the HttQ23-GFP a post-lysis-control was carried out in each experiment to verify the obtained aggregates were formed during the cell culture in living cells and not afterwards in the test tube. Briefly Q-peptides were added to a cell lysate of cells transfected with Htt-Q23-GFP (2 days after transfection) in a final concentration of 0.2 um. Hereafter the mixture was sonicated and treated in the same way as the samples.

Cell extracts and samples preparation

24 or 48 hours after transfection and Q-peptides addition the medium was washed away with PBS from the monolayer thoroughly in order to wash away the Q-peptides as much as possible. Cells were scraped in FTA-buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl) + 2% SDS and sonicated. Protein content was determined with the DC protein assay (Bio-RAD). Western blot samples were prepared at a final concentration of 1µg/µl in SDS-PAGE loading buffer and heated for 5 min at 100 °C. Filter trap samples were prepared at a final concentration of 100 ng/µl, 20 ng/µl and 4 ng/µl in FTA buffer with 2%SDS and 50 mM dithiothreitol and heated for 5 min at 100 °C. Samples were used immediately or kept frozen at -20°C.
**Western blot analysis**

Equal amounts of protein were loaded on 12.5% or 16% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and probed with mouse anti-GFP antibody JL-8 (Clontech) at a 1:5000 dilution, mouse anti-V5 antibody (Invitrogen) at a 1:5000 dilution. GAPDH was used as a loading control and was detected with a mouse antibody (RDI Research Diagnostics) at 1:10000 dilution. To visualize the Q-peptides detection was done on a 16% SDS-PAGE gel with a polyglutamine mouse monoclonal antibody 1:2000 (3B5H10, Sigma). This antibody does only react with soluble polyglutamine. Blots were subsequently incubated with HRP-conjugated anti-mouse secondary antibody (Amersham) at 1:5000 dilution and visualization was performed with Enhanced Chemiluminescence and Hyperfilm (ECL, Amersham). In most cases the stacking gel was analysed in parallel to detect the High Molecular Weight (HMW) fraction of aggregated HttQ23-GFP and Q-peptides.

**Filter trap assay**

To determine protein aggregates, the filter trap assay was performed as previously described (14). Briefly, 10, 2 and 0.4 µg of protein extracts were applied onto 0.2 µm pore Cellulose Acetate membrane prewashed with FTA + 0.1% SDS. Mild suction was applied and the membrane was washed 3 times. Aggregated proteins trapped in the membrane were probed with mouse anti-GFP antibody JL-8 (Clontech) at a 1:5000 dilution and mouse anti-polyglutamine antibody (Sigma) at a 1:5000 dilution followed by HRP-conjugated anti-mouse secondary antibody (Amersham) at 1:5000 dilution. Visualization was performed using enhanced chemiluminescence and Hyperfilm (ECL, Amersham).

Possible blocking of the cellulose acetate membrane by the Q-peptides and, as a result, preventing soluble HttQ23-GFP to pass through the membrane was checked by applying first a sample with Q-peptides and hereafter a sample with HttQ23-GFP. Only minor blockage was observed.

**Confocal microscopy**

24-48 hours after transfection and Q-peptides addition the medium was washed away with Phosphate-Buffered Saline (PBS) from the monolayer thoroughly and the cells were fixed with 3.7% formaldehyde for 15 minutes and hereafter were washed three times with PBS. To visualize nuclei, cells were stained 10 minutes with 0.2 µg/ml 4’,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted in Citifluor. Images of Alexa-550, GFP and DAPI fluorescence were obtained using the Leica confocal laser scanning microscope (Leica TCS SP2, DM RXE) with a 63X/1.32 oil lens. The captured images were processed using Leica Confocal Software and Adobe Photoshop.
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**SUPPLEMENTARY INFORMATION – CHAPTER 4**

Fig. S1: Western Blot assay of cell extracts at indicated time points and conditions.

Nitrocellulose membranes were probed with the polyQ antibody 1C2 as an additional check corresponding to Fig. 1 (A), Fig. 3 (B) and Fig. 4 (C).
DNAJB6 can’t prevent aggregation propagation

Chapter 4